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PRINCIPAL INVESTIGATOR: Frank K. Rauscher, Ph.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, Pennsylvania 19104

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Frank K. Rauscher, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
Philadelphia, Pennsylvania 19104

E-Mail: rauscher@wistar.upenn.edu

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Foreword

Progress is summarized on our project to investigate the diagnostic and therapeutic potential for Bin1 in prostate cancer. We have successfully generated 9 new monoclonal antibodies capable of recognizing all Bin1 splice isoforms that have been identified in cells. These antibodies are currently being tested and optimized for staining of fixed tissues. In Aim 2, we proposed to perform immunohistochemical studies. To date, we have analyzed 30 cases of frozen primary prostate cancer and found that 29/30 retained Bin1 expression in epithelial cells. Additional cases including metastatic cancers with follow-up information are pending completion of antibody testing. We believe this line of work will prove informative, because Northern analysis of primary and metastatic cancers confirms frequent expression in primaries but indicates universal losses in metastatic tumors. To date, gene status has been examined in 15 primary tumors. Using a heterozygous marker within the Bin1 gene we have documented loss of heterozygosity (LOH) in 6/15 of these tumors (40% LOH rate). However, the remaining alleles in tumors exhibiting LOH did not show alteration or perhaps only polymorphism rather than mutation. Metastatic variants of these tumors were not available to learn whether mutation had occured later in progression. This possibility is viable insofar as we have observed expression of normal Bin1 message in androgen-dependent LNCaP cells but misspliced messages that encode inactive polypeptides in androgen-independent PC3 and DU145 cells. Further analysis of Bin1 in metastatic tumors is planned as is an examination of promoter methylation status. Functional investigations indicate that LNCaP and PC3 are relatively susceptible to growth inhibition by Bin1 whereas DU145 is relatively insensitive. Adenoviral vectors for Bin1 are nonspecifically toxic in our hands. Therefore, as an alternate and more satisfying physiological approach, we hope to cross Bin1 "knockout" mice that have been developed to prostate tumor-prone transgenic animals, as a way to accurately gauge the significance of Bin1 loss in prostate cancer development and its mechanism of action in apoptosis.

Introduction

The genetic causes of prostate cancer remain largely unknown. One of the most common chromosomal abnormalities seen in tumors which have acquired invasive and metastatic potential is gain of chromosome 8p, where c-Myc is located (Bova and Isaacs 1996). Gains of 8q of are well-correlated with disease progression insofar as they are found in 85% of lymph node metastases and 89% of recurrent hormone refractive tumors (Cher *et al.* 1996; Van Den Berg *et al.* 1995; Visakorpi *et al.* 1995). c-Myc amplification or overexpression is found in many prostate tumors and is a likely progression marker (Buttyan *et al.* 1987; Fleming *et al.* 1986; Jenkins *et al.* 1997). Oncogenic activation of c-Myc by gene amplification delivers a powerful signal that is sufficient to drive cell cycle progression and malignant growth in many types of cells, including prostate cells (Thompson *et al.* 1989). However, in premalignant cells, c-Myc can also activate apoptosis such that its oncogenic activation is balanced by apoptotic penalty (Prendergast 1999). Therefore, malignant cells may escape this penalty by inactivating tumor suppressor functions. p53 is an important player but is likely irrelevant to this process in prostate cells because inactivation of p53 does not compromise c-Myc-mediated apoptosis in epithelial cells (Sakamuro *et al.* 1995; Trudel *et al.* 1997). Thus, loss or inactivation of molecules other than p53 should be considered.

c-Myc lies an intersection of two signaling networks which target its C-terminal DNA binding domain and its N-terminal transcriptional transactivation domain (Sakamuro and Prendergast 1999). Recent advances have led to the identification of a set of novel N-terminal-interacting proteins which constitute a second Myc network (Sakamuro and Prendergast 1999). One of these proteins, Bin1, is a ubiquitous adaptor protein which has features of a tumor suppressor (Elliott *et al.* 1999; Sakamuro *et al.* 1996) that has been linked to cell death and differentiation decisions (Sakamuro and Prendergast 1999). We hypothesized that Bin1 might be inactivated in prostate cancer because the human Bin1 gene maps to chromosome 2q14 (Negorev *et al.* 1996), within a region of chromosome 2q that is frequently deleted in metastatic prostate tumors (Brothman 1997; Cher *et al.* 1996) but where no tumor suppressor gene has been identified to date. Since c-Myc is frequently amplified in prostate carcinomas and Bin1 can suppress malignant transformation by c-Myc (Sakamuro *et al.* 1996), loss of Bin1 activity would eliminate a mechanism that can limit full oncogenic activation of c-Myc.

Progress on aims to examine the significance of Bin1 in prostate cancer follow.

Aim 1. Develop monoclonal antibodies that can detect Bin1 in fixed tissues.
Tasks 1, 2, and 3 were completed. Task 4 is in progress.

Aim 2. Perform an immunohistological analysis of Bin1 in staged primary tumors and metastases.
Task 1 to gather, catalog, and grade 30 frozen tumors was completed. Task 2 to accumulate fixed tumor sections of different grades is pending completion of Aim 1, Task 4. Tasks 3 and 4 to perform immunohistochemistry are partlially completed pending completion of Aim 1, Task 4.

Aim 3. Identify mutations in the Bin1 gene in androgen-independent tumor cells.
Tasks 1-4 to analyze the Bin1 gene in primary prostate tumors is partly completed. Task 5 to assay genetic mutations is deferred pending discovery of such mutations.

Aim 4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.
Tasks were changed following the determination that the adenoviral vectors in use were exhibiting nonspecific toxicity on prostate cancer cell lines in our hands. Colony formation assays using plasmid vectors (alternate strategy for Tasks 1 and 2) were completed. For an alternate to Task 3, which is to examine the in vivo role of Bin1, we plan to exploit a "knockout" mouse model that has been developed. Task 4 to examine the mechanism of apoptosis by Bin1 is in progress using cells isolated from "knockout" mice.

Body

Aim 1. Develop monoclonal antibodies that can detect Bin1 in fixed tissues.

Tasks 1,2,3 to develop a new set of monoclonal antibodies are completed. We have obtained nine (9) new antibodies that recognize 4 different epitopes in the N-terminal BAR domain of Bin1. This domain is found in all the various splice isoforms of Bin1 that we have been characterized in cells. The antibodies have been shown to be specific and effective for immunoprecipitation (5/9 antibodies), immunofluorescence (5/9 antibodies), and Western analyses (9/9 antibodies). Two of the antibodies obtained are quite avid, based on their ability to immunoprecipitate Bin1 in extraction buffers containing 0.1% SDS (i.e. RIPA conditions), and most give excellent signal-to-noise on Western analysis of Bin1 directly from cell extracts. We are currently carrying out Task 4, which is to test the ability of antibodies to effectively stain fixed prostate tissue sections containing normal and malignant cells (which previous antibodies did not do). Optimization of conditions for staining will follow. We anticipate this Task will be complete within 2-4 months.

Aim 2. Perform an immunohistological analysis of Bin1 in staged primary tumors and metastases:

Task 1 to gather, catalog, and grade 30 frozen tumors was completed. Task 2 to accumulate fixed tumor sections of different grades is pending completion of the antibody characterization for fixed tissue immunohistochemistry. However, this task will not take long to complete, when ready, as Dr. Tomaszewski has already cataloged a significant number of tumors and we have identified a commercial source of tissues with follow-up data available for limited cost. Tasks 3 and 4 to perform immunohistochemistry are partially completed and now published (Ge *et al.* 2000). We examined 30 cases of frozen primary prostate cancer. 29/30 were positive for nuclear staining of Bin1. Scoring of nests of malignant cells indicated that the level of Bin1 in malignant cells in the sections were actually slightly higher than in normal cells, for reasons that were unclear. The increase was at best 2-fold but statistical analysis of the scoring, which was done double blind by our collaborators Drs. Minhas and Tomaszewski, indicated it was significant. Unfortunately, as is the case now for cases of frozen prostate cancer, there were no metastatic variants of the tumors examined from patients to determine whether Bin1 levels were reduced during progression. We believe this is likely, however, given that Northern analysis indicates undetectable levels of Bin1 in 10/10 metastatic lesions (Ge *et al.* 2000) that were kindly provided by Dr. Peter Nelson (CaPCURE Tissue Consortium, University of Washington). Continuation of the immunohistochemical analysis of fixed archival material, where follow-up data are available, awaits completion of antibody characterization.

Aim 3. Identify mutations in the Bin1 gene in androgen-independent tumor cells.

Tasks 1-4 to analyze the state of the Bin1 gene in prostate cancer cells is essentially complete. We examined DNA isolated from 23 microdissected tumors provided by Dr. Tomaszewski to determine if they were heterozygous for a microsatellite marker that we identified in intron 5 of the human Bin1 gene. 19 tumors gave interpretable data and 15/19 were informative for the marker. 6/15 of the informative tumors exhibited loss of heterozygosity (LOH), giving a rate of 40% loss of the Bin1 gene. This finding was in excellent agreement with a study that identified LOH at a rate of 42% in the midsection of chromosome 2q, where Bin1 is located. All coding exons expressed in normal prostate cells were examined for mutations, at the remaining allele in the 6/15 tumors exhibiting LOH. We found no clear evidence of mutation. A potential polymorphism was possible (depending on splice site selection) at the exon 2-3 border, leading to L52Q alteration. All six tumor DNAs exhibited this feature making its significance unclear. These tumors were all primary tumors and therefore likely to be androgen-dependent. Analysis of androgen-independent tumors is pending.

In the meantime, we have completed a thorough examination of Bin1 status in the androgen-dependent cell line LNCaP and the androgen-independent cell lines PC3 and DU145. LNCaP is normal, expressing a normal level of wild-type Bin1 messages (-10 and -10-13 isoforms (Wechsler-Reya *et al.* 1997)). In contrast, PC3 and DU145 each expressed reduced levels of a misspliced polypeptide that is known to be a loss-of-function isoform (Ge *et al.* 1999; Ge *et al.* 2000). The missplice event is inclusion of exon 12A, which normally appears only in neuronal isoforms of Bin1, but has also been seen in melanoma (Ge *et al.* 1999). Although this missplice event is sufficient for loss of function PC3 also sustains a mutation in this polypeptide. In summary, both PC3 and DU145 have sustained loss of function in Bin1 whereas the androgen-dependent LNCaP cells have normal Bin1 function intact.

Aim 4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.

The research design in this aim has been altered due to our experience that the recombinant adenoviruses to be used exhibited unacceptable levels of nonspecific toxicity in our prostate cancer cell lines. In place of Tasks 1 and 2, we performed a colony formation assay to determine whether ectopic expression of Bin1 affected the growth potential of LNCaP, PC3, or DU145 cells. Bin1 was observed to inhibit colony formation in LNCaP and PC3 cells but had much less effect on DU145 cell growth.

Although inducible cell lines would be desirable to study the effect on LNCaP and PC3 cells in more detail, to date our experience has been that existing systems are too leaky and that stably transfected cells lose the ability to inducibly express the transgene (even in the absence of inducer). We are still attempting to overcome this issue.

In a parallel line of work, we have observed that ectopic expression of Bin1 engages an apoptotic-like cell death process that is caspase-independent (Elliott *et al.* 2000). Briefly, this process is characterized by cell shrinkage, substratum detachment, blebbing and vesiculation, and DNA degradation. Cells do not exhibit signs of necrotic death despite the lack of caspase activation. Moreover, neither Bcl-2 nor inhibition of the Fas death receptor pathway block Bin1-induced cell death. The process is specific at some level, because SV40 T antigen can inhibit cell death by Bin1, and the DNA degradation observed is blocked by the serine protease inhibitor AEBSF (which also inhibits apoptosis by c-Myc (Kagaya *et al.* 1997)). The T antigen connection is interesting, insofar as Bin1 can kill cells that lack p53 or Rb function, suggesting that T antigen interferes with Bin1 function at some level other than affecting p53 or Rb function. While the mechanism is yet unclear, we have developed a Bin1 "knockout" mouse model the study of which is providing a useful model to further unravel the mechanism of Bin1-dependent cell death.

We are altering Aim 4 to incorporate use of this 'knockout' system. One goal is to determine whether loss of Bin1 in prostate tissue promotes prostate tumor formation and/or progression. Prostate models are currently limited. Using a published method (Zhang *et al.* 2000), we aim to generate a prostate-specific Myc oncomouse and cross these mice to the Bin1 mice. The Myc oncomouse generated has been shown to progress to prostatic intraepithelial neoplasia (PIN) but not farther (R. Buttyan, Columbia University, pers. comm.). Our prediction is that loss of Bin1 will promote progression, possibly corroborating the role of Bin1 loss at later stages of prostate cancer development and providing a model for prostate tumorigenesis (which are greatly desired). A second goal of work in the 'knockout' system is mechanistic analysis. Preliminary work with Bin1 null fibroblasts has demonstrated that loss of Bin1 renders cells resistant to apoptosis by TNF, DNA damage, and other insults. Current effort to focus on the defective response to TNF, the signaling pathways of which have been worked out at a high level of detail, will greatly promote efforts to determine how Bin1 promotes apoptosis and how its loss may be important in prostate cancer development (where there is evidence that progression to TNF resistance parallels the progression to androgen independence)

Conclusions

We have developed a new set of antibodies that may be useful in pathological analysis of fixed tissues. Analysis of frozen tissues has revealed that Bin1 losses occur primarily at later stages of prostate cancer progression concomitant with the acquisition of metastatic potential. Genetic evidence of LOH in primary tumors is not associated with mutation at the remaining allele. Gene status in metastatic tumors remains to be examined. However, Northern analysis of such tumors revealed universal loss of Bin1 expression. Consistent with loss of Bin1 function in advanced tumors that have progressed to androgen-independence, we found that androgen-dependent LNCaP cells expressed normal Bin1 but that androgen-independent PC3 and DU145 cells sustained a loss-of-function due to missplicing. Continuing studies of the consequences of Bin1 expression in malignant cells and the mechanism by which Bin1 blocks the growth of prostate cancer cell lines has focused on use of a newly available Bin1 "knockout" mouse. Recent data indicates that loss of Bin1 elicits resistance to a variety of apoptotic stimuli relevant to prostate cancer progression, including resistance to TNF and DNA damage.

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Publications and manuscripts related to this grant (see Appendices for preprints and reprints)

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   This is our first but fairly extensive study of Bin1 status in prostate cancer.

2. Elliott, K., Ge, K., Du, W., and Prendergast, G.C. (2000). The c-Myc-interacting protein activates a caspase-independent cell death process. Oncogene, in press.

   This study presents an initial investigation of the mechanism of Bin1 action in malignant cells

3. Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene **18:** 3564-3573.

   This study presents assays to analyze functional significance of Bin1 alterations identified in CaP

4. Mao, N.-C., Steingrimsson, E., Duhadaway, J., Wasserman, W., Ruiz, J., Copeland, N.G., Jenkins, N.A., and Prendergast, G.C. (1999). The murine Bin1 gene functions early in myogenic differentiation and defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. Genomics **56:** 51-58.

   This paper offers the structure of mouse Bin1 gene leading to generation of the knockout mouse

5. Sakamuro, D., Duhadaway, J., Ewert, D., Crouch, D.H., and Prendergast, G.C. A necessary role for Bin1 in c-myc-mediated apoptosis. Manuscript under revision.

   Initial evidence implicating Bin1 in c-Myc-driven apoptosis. The findings suggest why Bin1 may be lost in tumor cells that overexpress c-Myc, like prostate cancer cells.

Presentations describing Bin1 in prostate cancer were made at the following meetings in 1998-99:

a. Annual Oncogene Meeting, June 1999, Frederick MD - poster presentation
b. 1999 90th Annual Meeting of the American Association for Cancer Research, Philadelphia PA
c. CaPCURE Meeting, September 1998, Incline Village NV - poster presentation

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George C. Prenderast, Ph.D.	Associate Professor
Daitoku Sakamuro, Ph.D.	Research Associate
Ai-Xue Liu, Ph.D.	Research Associate